The absolute rate of cholesterol biosynthesis in monocyte-macrophages from normal and familial hypercholesterolaemic subjects

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The true rate of cholesterologenesis in cultured monocyte-macrophages was determined from the incorporation of [2-¹⁴C]acetate into cholesterol, using the desmosterol (cholesta-5,24-dien-3 β -ol) that accumulated in the presence of the drug triparanol to estimate the specific radioactivity of the newly formed sterols. It was shown that this procedure could be successfully adapted for use with cultured monocytes despite the accumulation of other unidentified biosynthetic intermediates. In cells maintained in 20% (v/v) whole serum approx. 25% of the sterol carbon was derived from exogenous acetate. Cholesterol synthesis was as high in normal cells as in cells from homozygous familial hypercholesterolaemic (FH) subjects and accounted for 50% of the increase in cellular cholesterol. The addition of extra low-density lipoprotein (LDL) reduced cholesterol synthesis, apparently through a decrease in the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). When incubated in lipoprotein-deficient serum some cells did not survive, but those that remained showed a normal increase in protein content; the amount of cellular protein and cholesterol in each well did not increase and cholesterol synthesis was reduced by over 80%. HMG-CoA reductase activity fell less dramatically and the proportion of sterol carbon derived from exogenous acetate increased, suggesting that the low rate of cholesterologenesis with lipoprotein-deficient serum was due to a shortage of substrate. The results indicate that under normal conditions monocyte-macrophages obtain cholesterol from endogenous synthesis rather than through receptor-mediated uptake of LDL, and that synthesis together with non-saturable uptake of LDL provides the majority of the cholesterol required to support growth.

Human blood monocytes rapidly increase in size when maintained in culture in medium containing whole serum and develop many of the properties characteristic of mature macrophages (Johnson *et al.*, 1977; Musson *et al.*, 1980). During the period of greatest growth normal cells produce specific surface receptors that enable them to remove LDL from the medium and to use it as a source of free cholesterol (Knight & Soutar, 1982a). Under normal conditions the cells do not express their maximum capacity for LDL uptake (Soutar &

Knight, 1982) but have a high activity of HMG-CoA reductase (Knight *et al.*, 1983), which is commonly assumed to provide an indication of the rate of endogenous cholesterol synthesis. Monocytes from FH subjects cannot express active LDL receptors, yet grow and develop apparently normally without exhibiting a higher HMG-CoA reductase activity or a greater rate of incorporation of [¹⁴C]acetate into sterols than the normal cells (Knight *et al.*, 1983). These results suggest that endogenous synthesis is an important source of the cholesterol required to support the growth of the cells and that the supply derived from receptor-mediated uptake of LDL is of relatively little significance. They contrast with results obtained using cultured fibroblasts, which indicate that confluent or almost confluent monolayers of cells

Abbreviations used: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; FH, homozygous familial hypercholesterolaemia.

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prefer to obtain any cholesterol that they need from the uptake of LDL rather than from cellular synthesis (Goldstein & Brown, 1977).

Neither HMG-CoA reductase activity nor [^{14}C]-acetate incorporation can be taken as a reliable guide to the rate of cholesterol synthesis since they taken no account of limitations or changes in the supply of endogenous substrates or of the dilution of radioactive precursors and intermediates. Gibbons & Pullinger (1979) have developed a method, based on the accumulation of desmosterol (cholesta-5,24-dien-3 β -ol) in the presence of the drug triparanol, which provides an estimate of the specific radioactivity of the newly-formed cholesterol and allows the true rate of cholesterol synthesis to be determined. In this paper we show that the method can be adapted for use with monocyte-macrophages and present a comparison between the absolute rates of cholesterologenesis in normal and FH cells under standard culture conditions and in the presence of additional LDL. We have also studied the response of cellular cholesterol synthesis to preincubation with lipoprotein-deficient serum, which surprisingly produces a decrease in the HMG-CoA reductase activity in the cells (Knight *et al.*, 1983).

Materials and methods

Cells and lipoproteins

The methods used for the isolation of mononuclear cells and for the preparation of LDL (ρ 1.019–1.055 g/ml) and lipoprotein-deficient serum have been described previously (Knight & Soutar, 1982a; Soutar & Knight, 1982). Mononuclear cells were seeded at a density of approx. 3×10^6 cells/well in 24 mm multi-dish wells. The adherent monocytes were maintained at 37°C in RPMI-1640 medium (pH 7.4, Gibco Europe) containing 20% (v/v) fresh serum from normal donors. All incubations were performed in an atmosphere of CO_2 /air (1:19) in a humidified incubator. The medium was replaced on the third day after seeding and the experiments were performed 3–4 days later when each well contained approx. 150 μg of cellular protein.

Specific radioactivity of desmosterol

After the required preincubation, medium was removed and filtered through a Millipore filter (0.22 μm pore diam.). The cells were washed once with fresh RPMI-1640 medium and 0.5 ml of the filtered medium was replaced into each well. [2- ^{14}C]Acetate (sodium salt, approx. 15 d.p.m./pmol) was added to give a final concentration of 2.5 mM and the cells incubated at 37°C. After 3 h, 1 μl of a solution of 100 μM -triparanol in propane-1,2-diol was added to each well and the cells were

incubated at 37°C for a further 4 h. Medium was then removed, the cells were washed twice with ice-cold phosphate-buffered saline (Gibco, Europe) and lysed in a solution of 0.125% Triton X-100 in 0.1 M-potassium phosphate buffer, pH 7.5 (100 μl /well). The lysates were removed and each well was washed with a further 100 μl of the Triton solution. Lysates and washings from six identical wells were pooled and 50 μl samples taken for protein estimation. The specific radioactivity of desmosterol was then estimated using the methods developed by Gibbons & Pullinger (1977, 1979), which have recently been described in great detail (Pullinger & Gibbons, 1983). Briefly, the Triton extract was heated at 90°C for 2 min and then cooled and incubated for 30 min at 37°C with 0.18 unit of cholesterol oxidase and 0.15 unit of cholesterol esterase. After saponification, the non-saponifiable lipids were extracted into hexane and subjected to t.l.c. at 4°C on alumina impregnated with AgNO_3 , with a mixture of toluene and hexane (4:1, v/v) as developing solvent. The band of desmostenone (cholesta-4,24-dien-3-one) was located by radioautography and eluted with diethyl ether. Stigmasta-4,22-dien-3-one (50 ng) was added as internal standard. After preparation of the heptafluorobutyl enol esters, portions were assayed for radioactivity and the mass of the fluorinated desmosterol derivative was determined by gas chromatography using a pulsed ^{63}Ni electron capture detector.

Incorporation of [^{14}C]acetate into cholesterol

Cells were prepared and incubated as described above, except that triparanol was not added to the incubation medium. [$1\alpha,2\alpha(n)\text{-}^3\text{H}$]Cholesterol was added to the pooled lysate to assess recovery. After oxidation, saponification and argentation chromatography, the band of cholestenone (cholesta-4-en-3-one) was located and assayed for radioactivity by scintillation spectrometry. When required, unoxidized sterols were separated on silica gel with chloroform as developing solvent.

Assays

Protein was assayed by the method of Lowry *et al.* (1951) or, if Triton was present, by the method of Bensadoun & Weinstein (1976). For the measurement of DNA, cells were dissolved in 0.3 M-KOH and assayed by the method of Donkersloot *et al.* (1972). HMG-CoA reductase was assayed as described previously (Knight *et al.*, 1983). To assay the cellular content of cholesterol and cholesterol esters, cells were lysed in 0.125% Triton X-100 and [^3H]cholesterol was added to assess recovery. The lysate was extracted with chloroform-methanol (2:1, v/v; Folch *et al.*, 1957), and the cholesterol and esters separated by t.l.c. on

silica gel with hexane/diethyl ether/acetic acid (90:10:1, by vol.) as developing solvent. 5 α -Cholestan-3 β -ol was added as internal standard, the esters were hydrolysed and cholesterol assayed by g.l.c. with 1.5% SE30 on Chrom WHP as stationary phase.

Calculations

C₂₇ sterols contain 15 carbon atoms derived from C-2 of sodium acetate. Thus, if all the sterol were synthesized from exogenous [2-¹⁴C]acetate its specific radioactivity (d.p.m./pmol) would be 15 times that of the acetate (d.p.m./pmol). The ratio between the observed specific radioactivity of desmosterol and this maximum value gives the proportion of the newly synthesized sterol that was derived from [2-¹⁴C]acetate. Cholesterol synthesis (nmol/4h) was calculated as the incorporation of [¹⁴C]acetate into cholesterol (d.p.m./4h) divided by the specific radioactivity of desmosterol (d.p.m./nmol) estimated in cells from the same preparation incubated at the same time with the same [2-¹⁴C]acetate but in the presence of 0.2 μ M-triparanol.

The amount of cholesterol provided from the degradation of LDL (μ g/mg of DNA) was calculated from the rate of degradation of LDL protein, using the ratio of protein to DNA in the cells at that time and assuming a ratio of total cholesterol to protein of 2.0 (Soutar & Myant, 1979). Differences between means were tested for statistical significance using Student's *t*-test.

Materials

Constituents of tissue culture medium were obtained from Gibco Europe, Paisley, Scotland, U.K. and Linbro Multi-Dishes (12 $\times$ 24 mm) from Flow Laboratories, Irvine, Scotland, U.K. [2-¹⁴C]-Acetate (sodium salt; 40–60 Ci/mol), [26(27)-¹⁴C]-desmosterol (53 Ci/mol) and [1 α ,2 α (n)-³H]-cholesterol (40–60 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. [³H]Desmosterol was purified from rat-liver cells that had been incubated with [³H]acetate and 2 μ M-triparanol, by t.l.c. on alumina impregnated with AgNO₃ with toluene as developing solvent. [³H]Desmosterone and [¹⁴C]desmosterone were prepared from the corresponding desmosterol by oxidation and argentation chromatography as described above. Triparanol was the gift of Dr. B. Morgan, Beecham Research Laboratories, Tadworth, Surrey, U.K.

Results

Incorporation of [2-¹⁴C]acetate and the effect of triparanol

Monocyte-macrophages were incubated with [2-¹⁴C]acetate and 0.2 μ M-triparanol, lysed in 0.125%

Triton X-100 and the sterol esters hydrolysed with cholesterol esterase. After treatment with cholesterol oxidase to convert reactive C₂₇ sterols into the corresponding 4-en-3-one derivatives suitable for detection by electron capture, the lipids were subjected to t.l.c. on AgNO₃-impregnated alumina and the radioactive bands were detected by radioautography (Fig. 1). Cholestenone (cholest-4-en-3-one, *R_F* 0.39), the major radioactive band, was well separated from desmostenone (cholesta-4,24-dien-3-one, *R_F* 0.26) and the barely detectable band of unoxidized cholesterol (*R_F* 0.13). Squalene and related compounds remained close to the origin (*R_F* 0.05). Approx. 30% of the radioactivity applied was present in two bands that ran ahead of cholestenone, denoted as 'unknown 1' (*R_F* 0.50) and 'unknown 2' (*R_F* 0.60). The other minor bands (*R_F* 0.46, 0.44 and 0.33) were not studied further. Chromatography of unoxidized extracts on silica gel established that the unknown compounds were C₂₇ sterols and that C₂₉ + C₃₀ sterols contained less than 3% of the total radioactivity incorporated into non-saponifiable lipids. Lanosterol (5 α -lanosta-8,24-dien-3 β -ol), used as a marker for

Fig. 1. Argentation chromatography of radioactive non-saponifiable lipids from monocyte-macrophages incubated with [¹⁴C]acetate

FH cells were maintained for 7 days in medium containing 20% (v/v) whole serum and incubated for 7h with 2.5 mM-[2-¹⁴C]acetate. Triparanol (0.2 μ M) was added for the final 4h. After oxidation and saponification the non-saponifiable lipids were extracted and subjected to t.l.c. on alumina impregnated with AgNO₃. The Figure shows a radioautogram of one lane containing the sample and one containing a standard mixture of [¹⁴C]desmostenone and [¹⁴C]cholestenone.

C₂₉ + C₃₀ sterols, ran just ahead of cholestenone on AgNO₃-impregnated alumina, well separated from the desmostenone.

Fig. 2 shows the way in which the amount of radioactivity in the major bands changed as the concentration of triparanol in the incubation medium was increased. Radioactivity in the cholestenone and unknown 2 bands progressively fell. Low concentrations of triparanol increased the amount of radioactivity in both desmostenone and unknown 1, but whereas that in unknown 1 continued to rise as the concentration of drug increased, the amount of radioactive desmosterol reached a maximum and then decreased. Higher concentrations of triparanol increased the radioactivity in the squalene band and slightly decreased the total incorporation into non-saponifiable lipids. Thus the concentration of triparanol that produced an almost complete inhibition of the formation of cholesterol did not cause radioactivity to be trapped in desmosterol but in other intermediates apparently earlier in the biosynthetic pathway. Appreciable amounts of radioactive desmosterol only accumulated with much lower concentrations of the drug.

The distribution of radioactivity between the various bands observed in a number of experiments is shown in Table 1. Monocyte-macrophages were preincubated for 24h in standard culture medium containing 20% serum, in the same medium with additional LDL or in medium containing 10% lipoprotein-deficient serum. They were then incubated with [2-¹⁴C]acetate in the presence or absence of 0.2 μ M-triparanol. Preincubation with additional LDL reduced the incorporation of [¹⁴C]acetate into non-saponifiable

Fig. 2. Effect of triparanol on the incorporation of [¹⁴C]-acetate into non-saponifiable lipids by monocyte-macrophages

FH cells were maintained for 7 days in medium containing 20% (v/v) whole serum and incubated for 4h with 2.5mM-[2-¹⁴C]acetate and the indicated concentration of triparanol. After oxidation, saponification and argentation chromatography the amount of radioactivity in the various bands was determined. The values were obtained from nine wells of cells at each concentration and are shown for the bands (see Fig. 1) containing cholestenone (○), desmostenone (●), squalene (■), 'unknown 1' (▲) and 'unknown 2' (△) as well as for the unfractionated extract (▽).

Table 1. Effect of LDL, lipoprotein-deficient serum and triparanol on the distribution of radioactivity between non-saponifiable lipids

Cells were maintained for 6 days in medium containing 20% (v/v) whole serum and then for 20h in the same medium, medium with an additional 200 μ g of LDL protein/ml or medium containing lipoprotein-deficient serum (5mg of protein/ml). They were then incubated with 2.5mM-[2-¹⁴C]acetate for 7h, with triparanol (0.2 μ M) present, when required, for the final 4h. The bands obtained from argentation chromatography (see Fig. 1) were assayed for radioactivity. Over 90% of the radioactivity in the unfractionated non-saponifiable fraction was recovered in the six bands studied. Results with normal cells were not different from those with FH cells and the values shown are the means $\pm$ S.E.M. of results from four separate preparations of normal cells and three of FH cells.

Preincubation conditions	Triparanol	Incorporation of [¹⁴ C]acetate into total non-saponifiable lipids (nmol/mg of protein)	Proportion of non-saponifiable radioactivity in each band (%)					
			Origin	Squalene	Desmostenone	Cholestenone	Unknown 1	Unknown 2
Serum (20%)	—	11.06 $\pm$ 1.29	15.5 $\pm$ 1.4	4.4 $\pm$ 0.3	2.0 $\pm$ 0.4	59.5 $\pm$ 2.4	4.4 $\pm$ 0.6	13.6 $\pm$ 2.1
	+	10.73 $\pm$ 1.37	24.1 $\pm$ 3.3	7.1 $\pm$ 1.2	6.4 $\pm$ 1.1	33.1 $\pm$ 3.1	19.7 $\pm$ 2.8	9.5 $\pm$ 1.3
Serum (20%) + LDL	—	7.29 $\pm$ 1.21	18.1 $\pm$ 2.0	5.4 $\pm$ 1.4	3.7 $\pm$ 0.7	55.0 $\pm$ 3.3	5.7 $\pm$ 0.9	12.1 $\pm$ 2.0
	+	7.05 $\pm$ 1.04	25.4 $\pm$ 2.9	5.9 $\pm$ 0.7	6.5 $\pm$ 1.1	35.5 $\pm$ 2.8	16.6 $\pm$ 2.1	10.1 $\pm$ 0.9
Lipoprotein-deficient serum	—	8.36 $\pm$ 1.40	21.1 $\pm$ 3.5	11.3 $\pm$ 1.1	5.2 $\pm$ 0.7	25.5 $\pm$ 1.5	10.6 $\pm$ 1.7	25.2 $\pm$ 2.6
	+	9.24 $\pm$ 1.39	23.9 $\pm$ 4.5	15.2 $\pm$ 1.6	8.6 $\pm$ 1.0	19.8 $\pm$ 2.4	22.2 $\pm$ 3.8	12.3 $\pm$ 1.9

lipids but had no effect upon the way in which the radioactivity was distributed between the different compounds. Preincubation with lipoprotein-deficient serum, on the other hand, drastically reduced the proportion of the radioactivity present in cholestenone and correspondingly increased the proportion in the other bands without greatly affecting the relationship between them. Triparanol had no effect upon the incorporation of [^{14}C]acetate into total non-saponifiable lipids by the cells under any of the incubation conditions and in each case increased the proportion of the radioactivity present in unknown 1 while reducing the proportion in unknown 2. It decreased the radioactivity in cholestenone and increased that in desmostenone, although the effects were less marked in cells that had been preincubated with lipoprotein-deficient serum.

In the absence of triparanol it took 3–4 h from the addition of [^{14}C]acetate for the amount of radioactivity in the desmostenone and the two unknown bands to approach their maximum and for the rate of incorporation of radioactivity into cholesterol to become constant (Fig. 3). The addition of triparanol after 3 h produced an immediate reduction in the rate of incorporation into cholesterol and in the amount of radioactivity in the unknown 2 band, as well as an increase in the radioactivity present in the desmostenone and unknown 1 bands that was sustained for at least a further 4 h (Fig. 3).

Measurement of desmosterol mass and radioactivity

The radioactive compound that accumulates in the presence of triparanol and has the chromatographic properties of desmostenone after oxidation has been identified as authentic desmosterol by mass spectrometry and co-crystallization (Gibbons & Pullinger, 1977). In the present experiments the radiochemical purity of the radioactive band corresponding to desmostenone was investigated by t.l.c. The ^{14}C -labelled bands from a number of experiments were eluted with toluene and mixed with [^3H]desmostenone, prepared by oxidation of chromatographically pure [^3H]desmosterol as described in the Materials and methods section. Rechromatography on AgNO_3 -impregnated alumina gave a single band in which the $^{14}\text{C}/^3\text{H}$ ratio (0.184) was the same as the mixture applied (0.188), showing that the original desmostenone band was not significantly contaminated with radioactivity from the much larger mass of cholestenone. Chromatography of the mixture on silica gel, which separates compounds on the basis of polarity rather than content of double bonds, also gave a single band in which the $^{14}\text{C}/^3\text{H}$ ratio was only slightly higher (0.205) than that of the starting material. Thus there was no evidence of

Fig. 3. Time course of the incorporation of [$2\text{-}^{14}\text{C}$]acetate into sterols by monocyte-macrophages

FH cells were maintained for 7 days in medium containing 20% (v/v) whole serum and were incubated for up to 7 h with 2.5 mM [$2\text{-}^{14}\text{C}$]acetate. Triparanol ($0.2\text{ }\mu\text{M}$) was added after 3 h when required. Radioactivity in the bands obtained after argentation chromatography was determined. (a,b) Results are shown for the incorporation of radioactivity into the bands containing cholestenone (O), 'unknown 2' (Δ), desmostenone ($\bullet$) and 'unknown 1' ($\blacktriangle$). The solid line follows values from incubations without triparanol and the broken line follows values from incubations with triparanol. (c) The concentration ($\square$) and specific radioactivity ($\blacktriangledown$) of desmosterol were determined from the bands containing desmostenone obtained from incubations with triparanol. Each point represents the value from six wells of cells.

any major radioactive contamination of the desmostenone bands.

Analysis of the desmostenone band by g.l.c. showed a peak with an identical retention time as

desmosterone which could be used to calculate the mass and specific radioactivity of the desmosterol in the cells. On the addition of $0.2\ \mu\text{M}$ -triparanol the desmosterol content of the cells rapidly increased (Fig. 3c). If the drug was added later than the $[^{14}\text{C}]$ acetate at a time when the lag in incorporation into desmosterol and cholesterol was over, the increase in the mass of desmosterol almost matched the increase in radioactivity and its specific radioactivity quickly reached a maximum (Fig. 3c). Similar results were obtained with cells preincubated in lipoprotein-deficient serum, although the increase in the mass of desmosterol was not so great.

The monocyte-macrophages contained a small amount of desmosterol (approx. 25 ng/mg of protein) even in the absence of triparanol. Unfortunately, because of the limits of sensitivity of the assay, a reliable estimate of the mass under these conditions required too many cells to be practical as a routine experimental procedure, and triparanol was normally added to facilitate the measurement of the specific radioactivity of desmosterol. However, three experiments were performed in which enough cells were harvested to provide a comparison between values obtained in the presence and absence of the drug. In two experiments with cells maintained in 20% serum and one with cells preincubated in lipoprotein-deficient serum, the specific radioactivities with triparanol were 96%, 86% and 101% respectively of the corresponding values obtained without triparanol.

Effect of extracellular acetate

As the concentration of $[^{14}\text{C}]$ acetate in the incubation medium was raised the incorporation

Fig. 4. Effect of acetate concentration on the incorporation of $[^{14}\text{C}]$ acetate into cholesterol (O) and on the proportion of newly-synthesized sterol derived from $[^{14}\text{C}]$ acetate (▼)

FH cells were maintained for 7 days in medium containing 20% (v/v) whole serum and were incubated for up to 7 h with the required concentration of $[^{14}\text{C}]$ acetate. Incorporation into cholesterol was determined as the difference between 3 h and 7 h in the radioactivity of the cholestenone band (see Fig. 1) from cells incubated without triparanol. The proportion of sterol derived from acetate was calculated from the specific radioactivity of desmosterol measured in identical cells to which triparanol was added for the final 4 h of incubation. Cells from six wells were pooled for each point.

Table 2. Cholesterol synthesis in monocyte-macrophages from normal and FH subjects

Cells prepared from four different normal subjects and on two separate occasions from two different FH subjects (nos. 5 and 7 from one subject, 6 and 8 from the other) were maintained for 7 days in medium containing 20% (v/v) whole serum from a normal donor. $[2\text{-}^{14}\text{C}]$ Acetate was added to each well to give a final concentration of 2.5 mM . Six wells of each were incubated at 37°C for 3 h and six for 7 h. Lysates from the six wells were pooled and the radioactivity in the cholestenone band obtained after argentation chromatography was determined. Acetate incorporation was calculated from the difference in values obtained for each preparation at 3 h and 7 h. Triparanol ($0.2\ \mu\text{M}$) was added to a further six wells for the final 4 h of a 7 h incubation and the proportion of sterol derived from $[^{14}\text{C}]$ acetate was calculated from the specific radioactivity of the desmosterol that accumulated (see the Materials and methods section).

	Proportion of newly synthesized sterol derived from $[^{14}\text{C}]$ acetate (%)	Incorporation of $[^{14}\text{C}]$ acetate into cellular cholesterol (nmol/4h per mg of cell protein)	Amount of cellular cholesterol synthesized (nmol/4h per mg of cell protein)
Normal cells 1	28.9	5.34	1.23
2	22.1	4.60	1.37
3	21.8	2.10	0.64
4	19.7	2.41	0.82
Mean $\pm$ S.E.M.			1.02 ± 0.17
FH cells 5	24.3	3.84	1.05
6	35.5	7.62	1.43
7	28.6	4.02	0.94
8	21.3	4.39	1.37
Mean $\pm$ S.E.M.			1.20 ± 0.12

of radioactivity into cholesterol increased, rapidly at first and then approached a maximum (Fig. 4). The proportion of newly-synthesized cholesterol that was derived from [¹⁴C]acetate, calculated from the specific radioactivity of desmosterol, increased in an identical manner. Raising the concentration from 0.2 mM- to 2.5 mM-acetate led to an 82% increase in incorporation and an 84% increase in specific radioactivity in cells preincubated in 20% serum (Fig. 4) and to a 39% increase in each in cells preincubated in lipoprotein-deficient serum (results not shown). Thus under both conditions the rate of cholesterologenesis had remained constant and was not affected by the [¹⁴C]acetate present in the medium.

Cholesterologenesis in normal and FH cells

Monocyte-macrophages were incubated with 2.5 mM-[2-¹⁴C]acetate in the absence of triparanol. Because there was an appreciable lag before [¹⁴C]-cholesterol accumulated at a constant rate (Fig. 3a) some cells were harvested after 3 h and others after 7 h. The difference in the ¹⁴C content of the cholestenone bands was used to calculate the rate of incorporation of [¹⁴C]acetate into cholesterol. The specific radioactivity of desmosterol was determined at the same time in wells of identical cells to which 0.2 μM-triparanol had been added 3 h after the [¹⁴C]acetate, and the cells harvested 4 h later. Table 2 gives the results for separate preparations of normal and FH cells, each of which had been maintained in medium containing 20% serum. A comparison between the specific radioactivities of desmosterol and the acetate in the medium showed that approx. 25% of the newly formed cholesterol had been derived from the [¹⁴C]acetate and 75% from non-radioactive intracellular intermediates. The total amount of cholesterol synthesized from both sources was not significantly higher in FH cells than in normal cells (Table 2).

Effect of LDL and lipoprotein-deficient serum on cholesterologenesis

Each preparation of cells was divided into three groups, which were preincubated for 24 h in either standard medium containing 20% serum, medium with additional LDL or medium containing lipoprotein-deficient serum, before being assayed for cholesterologenesis and HMG-CoA reductase. Values for reductase activity are similar to those obtained previously (Knight *et al.*, 1983). Results from such direct comparisons with three preparations of normal cells and three of FH cells are shown in Table 3. Values obtained with FH cells were never significantly different from those with normal cells, so the results from all six preparations were combined. Preincubation with additional

Table 3. Effect of LDL and lipoprotein-deficient serum on cholesterol synthesis in monocyte-macrophages from normal and FH subjects
Cells were maintained for 6 days in medium containing 20% (v/v) whole serum and for a further 24 h in the same medium, medium with an additional 200 μg of LDL protein/ml or medium containing lipoprotein-deficient serum (5 mg of protein/ml). Cholesterol synthesis and the proportion of sterol derived from [¹⁴C]acetate were determined as described in Table 2. [¹⁴C]Cholesterol in the medium was assayed in the same way as that in the cells. HMG-CoA reductase activity was determined with cells from duplicate wells. Values shown are the means ± s.e.m. of results from three separate preparations of normal cells and three of FH cells. *Significant (*P* < 0.05) effect of LDL, **significant (*P* < 0.05) effect of lipoprotein-deficient serum.

Preincubation conditions (20%)	Cells	Proportion of newly-synthesized sterol derived from [¹⁴ C]acetate (%)	Amount of newly synthesized cholesterol (pmol/4h per mg of protein)		HMG-CoA reductase (nmol/2h per mg of protein)
			Present in cells	Present in medium	
Serum (20%)	Normal	24.2 ± 2.3	1019 ± 227	284 ± 82	20.0 ± 1.8
	FH	29.4 ± 3.2	1185 ± 148	262 ± 60	18.4 ± 2.6
Serum (20%) + LDL	Normal	22.2 ± 4.8	686 ± 179	106 ± 4	10.0 ± 2.5
	FH	26.9 ± 1.4	627 ± 47	132 ± 34	7.2 ± 2.0
Lipoprotein-deficient serum	Normal	41.0 ± 8.5	168 ± 18	42 ± 16	12.6 ± 1.7
	FH	39.9 ± 9.8	184 ± 30	70 ± 26	12.3 ± 1.7

Table 4. *Protein and cholesterol content of monocyte-macrophages*

Cells were maintained for 6 days in medium containing 20% (v/v) whole serum. They were then preincubated for 24h in the same medium, medium containing an additional 200 μ g of LDL protein/ml or medium containing lipoprotein-deficient serum (5mg of protein/ml). Duplicate wells of cells were dissolved in 0.1M-NaOH to measure protein, 0.3M-KOH to measure DNA or 0.125% Triton X-100 to measure cholesterol and cholesteryl esters. Values for normal cells were the same as those for FH cells and are shown as means $\pm$ S.E.M. of results from two of the preparations of normal cells and two of the preparations of FH cells used to obtain the results in Table 3. *Significant ($P < 0.05$) decrease with lipoprotein-deficient serum.

Preincubation conditions	DNA/well (μ g)	Protein content (μ g/ μ g of DNA)	Cholesterol content (μ g/mg of protein)	
			Unesterified	Esterified
Before incubation	5.37 $\pm$ 0.91	24.0 $\pm$ 0.9	21.2 $\pm$ 1.5	3.8 $\pm$ 0.7
Serum (20%)	5.44 $\pm$ 0.99	34.4 $\pm$ 1.3	22.1 $\pm$ 2.1	3.1 $\pm$ 0.2
Serum (20%) + LDL	5.11 $\pm$ 1.02	33.8 $\pm$ 2.1	26.8 $\pm$ 1.2	2.6 $\pm$ 0.4
Lipoprotein-deficient serum	3.64 $\pm$ 0.76*	36.7 $\pm$ 1.1	19.6 $\pm$ 0.4	2.7 $\pm$ 0.8

LDL (200 μ g of protein/ml) caused a similar reduction in HMG-CoA reductase activity and in the amount of cholesterol synthesized by the cells without affecting the proportion of newly-synthesized sterol derived from [14 C]acetate. After preincubation with lipoprotein-deficient serum, [14 C]-acetate provided a greater proportion of the cholesterol synthesized. However, the rate of cholesterol synthesis was decreased dramatically and by much more than the activity of HMG-CoA reductase. This could not be explained by any increase in secretion of newly-formed cholesterol. Approx. 25% of the newly-synthesized cholesterol was released by the cells and this was not significantly affected by the composition of the medium (Table 3).

Protein and cholesterol content of the cells

Between the sixth and seventh day after seeding the protein content of the cells increased by 40% and the ratio of unesterified cholesterol to protein remained constant (Table 4). Additional LDL had no effect on the development of the cells, nor on the content of cholesteryl ester, which remained low. Incubation in lipoprotein-deficient serum resulted in a loss of adherent cells, as indicated by the decrease in DNA content of the wells. However, the cells that remained had developed to the same extent as those incubated with lipoproteins (Table 4). Interestingly, the amount of cellular protein and cholesterol in each well stayed the same during incubation with lipoprotein-deficient serum, suggesting that the loss that would be expected from the reduction in DNA content matched the increase in protein and cholesterol in the cells that remained.

Discussion

It is not possible to measure directly the mass of cholesterol synthesized by cells since the amount

formed is much smaller than that already present in cellular membranes. Furthermore the unknown dilution of [14 C]-labelled precursors by endogenous substrates prevents any reliable assessment of the true rate of synthesis merely from the incorporation of radioactivity into cholesterol. The use of $^3\text{H}_2\text{O}$ as substrate overcomes some of the problems in certain tissues but there are theoretical uncertainties in applying this method to different types of cell that may derive acetyl-CoA from different precursors and practical difficulties in using it with small numbers of cells in open dishes. Gibbons & Pullinger (1977) have shown that inhibition of Δ^{24} -bond reduction in liver cells by the addition of triparanol resulted in a decrease in the incorporation of [14 C]acetate into cholesterol and an accumulation of a corresponding amount of radioactive desmosterol. After oxidation and purification the small mass of desmosterol could be assayed by g.l.c. using an electron-capture detector, and the specific radioactivity determined. Since desmosterol is produced late in the cholesterol synthetic pathway its specific radioactivity can be assumed to be the same as that of the newly formed cholesterol. We have shown in the present paper that this procedure can be successfully applied to cultured monocyte-macrophages, despite the accumulation of other biosynthetic intermediates. With a low concentration of triparanol, which did not affect the overall incorporation of [14 C]acetate into non-saponifiable lipids, enough desmosterol accumulated to give a reliable estimate of its mass and specific radioactivity. This could then be used to calculate the true rate of cholesterogenesis from the constant rate of incorporation of [14 C]acetate into cholesterol in corresponding cells incubated without triparanol.

The presence of a significant amount of radioactivity in the two unknown compounds indicates that at least one enzyme along the biosynthetic pathway in monocyte-macrophages has a rela-

tively low activity. However it does not seem to have any particular regulatory function since, after allowing for alterations in specific radioactivity, there is little change in the cellular content of the unknown compounds under different incubation conditions (Table 1). We have not attempted any detailed identification of unknown 1 and unknown 2. Both run on t.l.c. as C_{27} sterols and are earlier intermediates than desmosterol in the synthetic pathway. Unknown 1 appears to accumulate at the expense of unknown 2 on the addition of triparanol, suggesting that they are closely related and linked through the reduction of the Δ^{24} -bond.

Between the sixth and seventh day after seeding, cells maintained in medium containing 20% whole serum increased their content of cholesterol by some 230 $\mu\text{g}/\text{mg}$ of DNA (Table 4). The rate of cholesterol synthesis by these cells, calculated from the results in Table 3 and Table 4, was approx. 110 μg of cholesterol/mg of DNA per day, or about one-half of the amount required. Our previous results have shown that non-saturable uptake of LDL by normal cells (Knight & Soutar, 1982a) was similar to that of FH cells (Soutar & Knight, 1982; Knight *et al.*, 1983) and could provide approx. 80 μg of cholesterol/mg of DNA per day (see the Materials and methods section). Thus the cells could have acquired most of the cholesterol they needed from direct synthesis and non-saturable uptake of LDL. Any shortfall could easily be made up in normal cells through receptor-mediated uptake of LDL, which, from previous experiments, would provide about 90 μg of cholesterol/mg of DNA per day. In the present experiments FH cells developed at the same rate as normal cells and their rate of cholesterol synthesis was slightly, although not significantly, higher. It is clear that cholesterol synthesis in FH cells had not increased enough to replace receptor-mediated uptake of LDL. However, since the rate was high even in normal cells, we cannot, from the current results, exclude the possibility that synthesis was increased in FH cells by the small percentage needed to make up the cholesterol requirement for growth.

The addition of an extra 200 μg of LDL protein/ml would provide an extra 120 μg of cholesterol/mg of DNA per day through non-saturable uptake. As well as a decrease in receptor-mediated uptake of LDL (Fogelman *et al.*, 1982; Knight *et al.*, 1983) this led to a decrease in the rate of cholesterol synthesis which appeared to result entirely from a fall in the activity of HMG-CoA reductase. The effect of preincubation with lipoprotein-deficient serum could not be explained by such a mechanism. Cholesterol synthesis fell almost to zero whereas reductase activity was only reduced by 40%. There was no particular redistribution of radioactivity between inter-

mediates that would indicate that there had been a regulatory inhibition of some step in the biosynthetic pathway. Furthermore, the proportion of newly-synthesized cholesterol that had been derived from [^{14}C]acetate was greatly increased, suggesting that the low rate of cholesterol synthesis resulted from a lack of substrate supply rather than from direct enzymic control. It might be expected that cholesterol synthesis in the cells would increase to compensate for the removal of lipoprotein cholesterol from the medium. In fact this did not occur and the amount of both cholesterol and protein in each well remained constant. Surprisingly, through a decrease in DNA content, the ratio of protein to DNA in the wells increased to the same extent as that of cells maintained in whole serum. Thus some cells developed normally while others were degraded, with the clear implication that the surviving cells utilized the degradation products to sustain their growth.

From experiments with confluent or almost confluent cultured fibroblasts, it has been suggested that cells, whenever possible, obtain any cholesterol that they require through receptor-mediated uptake of LDL (Goldstein & Brown, 1977). This did not appear to be the case in developing monocyte-macrophages. Although normal cells had the ability to produce enough LDL receptors to satisfy their need for cholesterol without endogenous cholesterol synthesis, they did not express their maximum number of receptors (Knight & Soutar, 1982b) and synthesized as much cholesterol as did FH cells. On the other hand, FH cells, and presumably normal cells, could develop perfectly adequately using only cholesterol from endogenous synthesis and non-saturable uptake, yet normal cells did exhibit some saturable, receptor-mediated degradation of LDL. There is obviously a complex inter-relationship between the mechanisms that regulate the supply of cholesterol within the cells, which, in cultured monocytes at least, favours the endogenous synthesis of cholesterol rather than the production of receptors for the uptake of LDL.

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